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Neuromuscular Junction in a Microfluidic Device

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Abstract—**Malfunctions at the site of neuromuscular junction (NMJ) of post-injuries or diseases are major barriers to recovery of function. The ability to efficiently derive motor neurons (MN) from embryonic stem cells has indicated promise toward the development of new therapies in increasing functional outcomes post injury. Recent advances in micro-technologies have provided advanced culture platforms allowing compartmentalization of sub-cellular components of neurons. In this study, we combined these advances in science and technology to develop a compartmentalized in vitro NMJ model. The developed NMJ system is between mouse embryonic stem cell (mESC)-derived MNs and c2c12 myotubes cultured in a compartmentalized polydimethylsiloxane (PDMS) microfluidic device. While some functional in vitro NMJ systems have been reported, this system would further contribute to research in NMJ-related diseases by providing a system to study the site of action of NMJ aimed at improving promoting better functional recovery.**

I. Introduction

The NMJ connects efferent nerves to muscle cells through a synapse, and is responsible for coupling neuronal action potential to muscle cell excitation. Disorders of the NMJ result from the absence or malfunction of the key proteins involved in neuromuscular transmission, most commonly through antibody mediated damage or downregulation of ion channels or receptors. Malfunctioning NMJs result in improper signaling and loss of muscle control, which in severe cases can lead to motor paralysis [1]. Amyotrophic lateral sclerosis (ALS) is an example of a motor neuron disease where malfunctioning of the NMJ contributes to loss of function. In this neurodegenerative disease, motor neurons slowly degrade, causing muscle atrophy from lack of use [2]. Animal models of ALS suggest that motor neuron death starts with NMJ destruction, which is followed by distal axon degeneration. In another example, spinal cord injury can cause a traumatic loss of motor neurons, with downstream consequences in remaining NMJs similar to NMJ defects observed in other neurodegenerative and injury-associated damage to motor neurons. Currently, therapies are aimed at slowing down the progression of these types of neurodegenerative diseases or limiting the extent injury, primarily in animal models. There is a focus on treatment rather than a cure. A growing avenue towards novel treatments is focusing beyond limiting the injury and towards promoting plasticity and regeneration in order to enhance functional recovery [8, 9]. The development of compartmentalized in vitro systems that recreate NMJs in culture will enable the investigation of treatments in repairing malfunctioning NMJs. Motor neuron cell bodies are located in the ventral horn of the spinal cord, while NMJs are located at the distal ends of their corresponding axons, potentially meters away. Several potential treatments, such as growth factors, may cause extensive complications when delivered systemically, and hence, localized treatment is a more viable option. Compartmentalized culture systems allow the ability to determine the site of action on the efficacy of such treatments within a more segregated environment as observed in vivo. Successful formation of functional NMJs has been achieved in culture, but primarily, these were achieved with cells co-cultured in the same in vitro environment [1]. These include multiple motoneuron and muscle co-cultures reported for Xenopus [12,13], chick [14-16], mouse [17,18] and rat [19]. There have also been cross-species experiments with chick muscle and mouse MN [18,21], as well as with human embryonic stem cell-derived MNs and C2C12 myotubes. Recently, Guo et al. demonstrated an in vitro NMJ system between human ESC- derived MNs and human ESC-derived skeletal cells [10]. As motor neurons and muscle cells experience very different microenvironments in vivo, we believe that culturing neurons and myoblasts in separate microenvironments, where only the axon passes between the environments, recapitulates the in vivo environment much more closely. Campenot chambers have previously been used with success but these systems showed poor reliability, reproducibility, challenges to manipulation, and a lack of a practical high thru put platform [1]. In this study we utilized a novel PDMS microfluidic device [3, 11], which addressed many of the obstacles faced by the Campenot chambers.

We incorporated mESC-derived MNs in our NMJ model. MNs are notoriously difficult to culture in vitro for extended periods of time in dissociated cultures. ESCs offer exciting and powerful genetic tools for scientific discovery [4]. These cells can be differentiated efficiently into all neural lineages, and such cells appear to be identical to primary cells [5-7]. This provides a reproducible system for cultured MNs. The conditions, growth factors, and transcription factors governing MN production are well established [4]. Importantly, ESCs offer knock in, knock out and conditional genetic systems [4]. In this study, we sought to develop a novel NMJ system that achieves compartmentalization between the MN somas and myoblasts and thus the NMJ by combining the tools of mESC-derived MNs and the microfluidics. This system would aid not only research related to NMJ-related diseases, but provide a novel system that reveals the site of action of NMJ for closer studies.

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II. Methods

A. Microfabrication and Preparation of Microfluidic Compartmentalized System

A novel PDMS microfluidic device was designed and fabricated as described previously [11]. To briefly illustrate, Fig. 1 demonstrates the replica molding of our device through soft lithography. The final product consisted of 100 parallel microchannels, each with dimensions: width=10 μm, length=500 μm, height = 2.5 μm, that divided two cellular compartments: neuron soma and muscle compartments, each with dimensions: width = 12.75 mm, length = 6.35 mm, height = 4.76 mm. These resulting devices were cleaned, plasma bonded to cover glass, and autoclaved. The channels were cleared by performing washes of the bonded devices with ethanol followed by water. Prior to loading C2C12 myoblasts, both compartments of PDMS platforms were coated with PDL overnight at room temperature and Matrigel for 1 hr at 37°C.

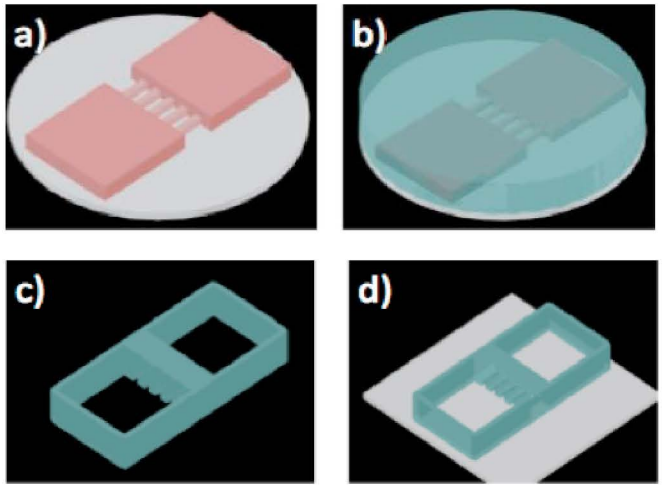

Figure 1. a) Microchannels and compartments were createdon a silicon wafer via microfabrication using SU-8 2002 and SU-8 3050(Microchem; MA). b) Mold was created by soft lithography using Sylgard 184 polydimethylsiloxane (PDMS). c) Excess PDMS and compartments were cut out. d) PDMS was plasma bonded to a coverslip.

B. C2C12 Culture and In Vitro Differentiation

Mouse myoblasts (C2C12) were cultured in 15% FBS (in DMEM). After loading and proliferation to confluence, C2C12 myoblasts were induced to differentiate into multinucleated myotubes in 2% HS (in DMEM).

C. mESC Culture and In Vitro Differentiation

Mouse ESCs (ROSA26) were cultured as previously shown [23] in ESC growth medium (Table 1). ESCs were induced in aggregate culture to produce embryoid bodies (EBs) (2-/6+ RA and Pur) in DFK-10 medium (Table 1) for 8 days [24].

D. Cell Loading and Neuron and Muscle Co-Culture in the Microfluidic Platform

First, 4 days before plating EBs, C2C12 myoblasts were plated at 20 cells/mm^2 in the muscle compartment. After culturing to confluence in ESC growth media, a mixture of 15% Fetal Bovine Serum (in DMEM), differentiation media, 2% Horse Serum (in DMEM), was applied for myotube differentiation. On day 8 of induction, 6 (2-6+ RA,Pur) EBs were loaded into the neuron soma compartment close to the microchannels. MNs were differentiated in neuron culture medium. When axons crossed microchannels, both sides were cultured in neuronal culture medium until the formation of NMJs.

D. Immunocytochemistry

About a week after axons crossed the microchannels, the cell cultures and motor neuron-enriched EBs were fixed with 4% paraformaldehyde. Immunocytochemistry was performed using standard protocols. All primary antibodies and bungarotoxin (BTX-555, Invitrogen) were administered overnight in 5% serum and PBS at 4°C and the secondary antibodies were applied in PBS for 1 hr at RT.

III. Results

Before loading the cells, a timetable was created, taking into account of the different timings of growth and differentiation. To be most efficient, the axon terminals must reach the myotubes shortly after the myotubes become mature and ready to form NMJs. As it takes 8 days for the mESCs to become MN-enriched EBs, and 1 day for axons to reach the muscle, we expected axon to reach the myotubes on day 9. Also, as it takes 1 day to grow C2C12 myoblasts to confluence and 4 days to fully differentiate them into myotubes, we calculated the plating of C2C12s to be 4 days before loading of the day 8 EBs. One day prior to plating the C2C12s, both compartments were coated with poly-D-lysine (PDL) overnight at room temperature, and one hour before loading, Matrigel was additionally coated at 37°C. The combination of PDL and Matrigel proved optimal for the growth of both cell types.

C2C12s successfully differentiated by the time loaded EBs stretched their axons across the channels (Fig. 2). Upon axons crossing the channels, the muscle compartment media was replaced with 50% C2C12 differentiation medium (2% HS) and 50% neuron culture medium; the MN compartment was kept in neuron culture medium. The neuron culture medium was substituted in order to provide growth factors for the growing axons in the muscle compartment. Further co-culture was maintained by changing both compartments' corresponding media every other day for a week.

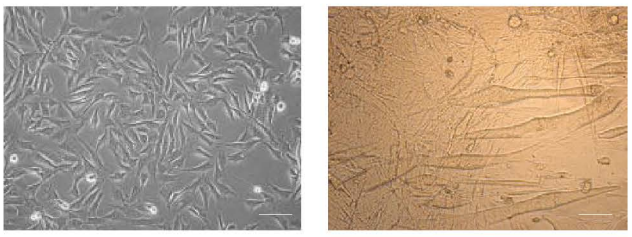

Figure 2. Differentiation of C2C12 myotubes. C2C12 myoblasts were grown to confluence in 15% FBS. Once 100% confluent, 2% HS media was applied. After 4 days fully differentiated myotubes were seen. The image on the right shows the mature myotubes in the microfluidic compartment. Scale bars = 50 μm.

Fig. 3 shows the phase contrast image of a representative co-culture 7 days after axons reached the myotubes. Microchannels, which only allow axons to pass through, divide the two compartments. In the muscle compartment, strands of myotubes are seen throughout the compartment. In the MN compartment, many axons are stretching out of motoneuron-enriched EBs.

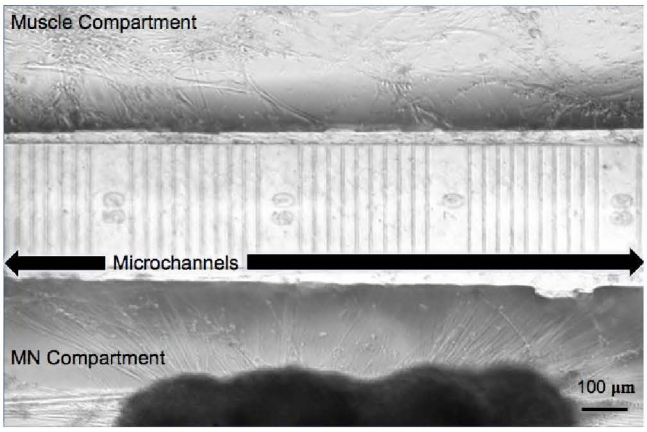

Figure 3. Phase Contrast Image of Co-Culture. This shows axons stretching out from MN-enriched EBs, across the microchannels, and into the muscle compartment with strands of myotubes. Scale bar = 100 µm.

Figure 4 shows a stained image of co-culture 7 days after axons touching the myotubes. The white arrows point to areas where axons from the MN compartment touch and anchor on parts of myotubes. These anchoring areas proved to be NMJs upon zooming at higher magnitude.

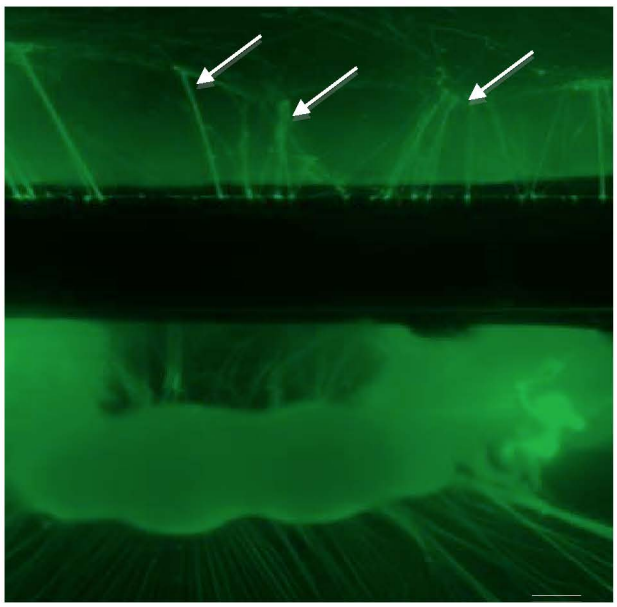

Figure 4. NF (green) staining shows axons crossing the microchannels and anchoring at surfaces of different myotubes. White arrows show potential sites of NMJ. Scale bar = 100 µm

The presence of NMJs was confirmed by the co-immunostaining of neurofilaments (green) and BTX-488 (alpha-bungarotoxin, Alexa Fluor 488 conjugate), which binds to acetylcholine receptors (AChR). The clustering of AChR where the axon terminals co-localized confirmed the formation of NMJ. Figure 6b shows double staining of BTX-488 and NF. The first set clearly demonstratess axons terminating at points on myotubes where clustering of AChR also occurred (Figure 5).

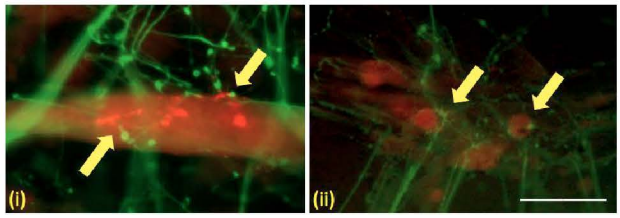

Figure 5. BTX (red) and NF (green) staining reveals termination of axons at various clusters of AChRs. (i) shows axons terminating upon reaching myotubes (yellow arrows). The second close up image, (ii), also shows axon terminals at clusters of AchRs as well as axons wrapping around the myotube. Scale bar = 50µm.

IV. Discussion

This study reports the first of its kind compartmentalized NMJ in vitro culture model. We utilize mESCs in this system in order to have a reliable source of dissociated MNs, and demonstrate their successful differentiation, as well as the differentiation of myoblasts into myotubes, within our compartment system. Through immunocytochemistry we showed indications of successful NMJ formation between these two segregated cells. Although a number of functional NMJ in vitro culture systems have been reported, this compartmentalized system more faithfully mimics the isolated in vivo environment while also allowing the site of action studies for treatments for NMJ-related diseases.

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